

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:

Iwao KATSUYAMA et al.

Application No.: 10/526,369

Filed: March 3, 2005

For: METHOD OF SCREENING  
PHYSIOLOGICALLY ACTIVE SUBSTANCE

Confirmation No.: 6217

GROUP: 1645

EXAMINER: Archie,  
Nina

DECLARATION UNDER 37 C.F.R. 1.132

Commissioner for Patents  
P.B. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Masao Tokunaga, Ph.D., residing in Kagoshima-ken, Japan, hereby declares and states as follows:

1. That I am one of the co-inventors of U.S. Application Serial No. 10/526,369 filed on March 3, 2005, entitled METHOD OF SCREENING PHYSIOLOGICALLY ACTIVE SUBSTANCE. I am thoroughly familiar with the contents of said Application, its prosecution before the United States Patent and Trademark Office and the references cited therein.
2. That I am a graduate of Graduate School of Osaka Prefecture University, Department of Agriculture, received a doctorate in the year 1976, majoring in agricultural chemistry.
3. That I have been extensively engaged in the field of biotechnology for at least 30 years, and is a professor at the Laboratory of Applied and

Molecular Microbiology, Faculty of Agriculture, Kagoshima University since 1993.

4. That the following experiments were conducted under my supervision and control in order to verify that a method of screening a physiologically active substance using a respiration ability-deficient strain of yeast of the present invention is technologically distinguishable from the method of screening described in Bounaga (WO 01/20020) using a wild type strain of yeast.

#### EXPERIMENTAL METHODS

A test was carried out by allowing two strains, i.e. a wild type strain and a respiration ability-deficient strain of yeast, to be in expressed states of heterogeneous proteins PARP1, and comparing the growth rate in the presence of a selective inhibitor NUI025 of PARP1.

##### 1. Strains Used

Wild-type strain: *S. cerevisiae* YPH500

Respiration ability-deficient strain: *S. cerevisiae* YPH500-12

##### 2. Media Used and Reagents

The media used and reagents are the same ones as those mentioned in the Declaration filed May 29, 2007.

##### 3. Transformation of Yeasts

The yeasts were transformed in the same manner as described in the Declaration filed May 29, 2007.

4. Culture Conditions

The culture conditions were the same as those described in the Declaration filed May 29, 2007, except that the final concentrations of the PARP1 inhibitor NU1025 were 0, 30 nM, 100 nM, 300 nM, 1  $\mu$ M, 3  $\mu$ M, 10  $\mu$ M, 30  $\mu$ M, 100  $\mu$ M, and 300  $\mu$ M.

EXPERIMENTAL RESULTS:

(1) The screening method using a wild-type strain and a respiration ability-deficient strain of an yeast, each strain being in expressed state of PARP1, was technologically evaluated by generating a dose-response curve against a concentration of NU1025 ( $-\log M$ ), assuming that the maximum OD value during 48 hours of culturing the cell strain is 100.

The growth rate of the wild-type strain of yeast in expressed state of PARP1 in the presence of NU1025 is as shown in Table A and FIG. I. On the other hand, the growth rate of the respiration ability-deficient strain in expressed state of PARP1 in the presence of NU1025 is as shown in Table B and FIG. II.

Incidentally, in a culture test of a wild-type strain and a respiration ability-deficient strain of an yeast in non-expressed state of PARP1 which was carried out at the same time, in both cases, any influences in the growth of the strains were not observed by the addition of NU1025.

Table A Effect of Inhibiting Growth of Wild-type Strains in Expressed State of PARP1 Protein in the Presence of NU1025

Strain	NU1025 (-Log M) / % of Maximum effect								
	7.5	7.0	6.5	6.0	5.5	5.0	4.5	4.0	3.5
①	4.3	3.8	1.9	17.7	35.9	60.5	81.3	100.0	96.7
YPH500 ②	1.1	10.5	24.8	53.8	60.4	80.9	90.9	98.0	100.0
③	-1.6	1.6	3.6	19.4	37.9	67.2	89.5	100.0	99.7

Table B Effect of Inhibiting Growth of Respiration Ability-Deficient Strains in Expressed State of PARP1 Protein in the Presence of NU1025

Strain	NU1025 (-Log M) / % of Maximum effect									
	7.5	7.0	6.5	6.0	5.5	5.0	4.5	4.0	3.5	
YPH500-12	①	1.0	1.6	6.3	12.7	24.6	39.9	62.2	94.4	100.0
	②	0.2	2.2	5.0	12.0	23.4	39.1	60.4	91.3	100.0
	③	0.7	2.3	6.5	14.4	25.4	41.9	62.7	94.1	100.0

FIG. I Effect of Inhibiting Growth of Wild-type Strains in Expressed State of PARP1 Protein in the Presence of NU1025

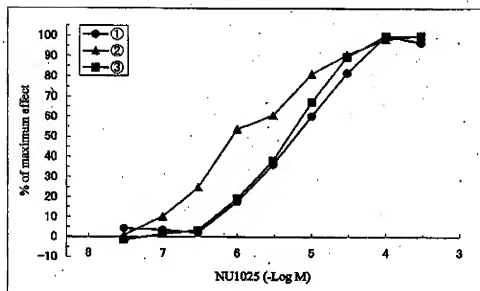
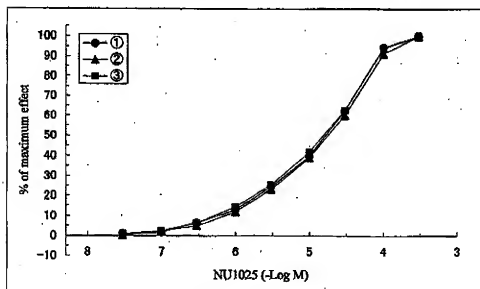


FIG. II Effect of Inhibiting Growth of Respiration Ability-Deficient Strains in Expressed State of PARP1 Protein in the Presence of NU1025



(2) Further, from the above results, 50% growth recovery effective concentration ( $EC_{50}$ ), an average thereof, standard error, and coefficient of variation (CV value) for NU1025 against the inhibition of growth of the yeast caused by PARP1 were obtained. The results are shown in Table C.

Table C  $EC_{50}$ -Values of NU1025 in the Wild-type Strains and the Respiration Ability-Deficient Strains in Expressed State of PARP1 Protein

Strain		$EC_{50}$	Average	SD	CV(%)
YPH500	①	7.1	4.7	2.8	60.6
	②	1.6			
	③	5.4			
YPH500-12	①	16.3	16.5	1.2	7.3
	②	17.8			
	③	15.5			

## DISCUSSION

As described above, in a case where a wild-type strain of an yeast in expressed state of PARP1 is used, the concentration of NU1025 necessary for 50% recovery of the growth inhibition by PARP1 is  $4.7 \pm 2.8 \mu\text{M}$ , and a coefficient of variance therefor is 60.6%. It is evident from the above facts that in the method of screening a physiologically active substance using a wild-type strain of an yeast, the experimental data obtained show a large experimental error, which makes them markedly unreliable.

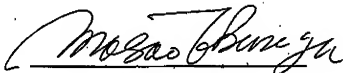
On the other hand, in a case where a respiration ability-deficient strain of an yeast in expressed state of PARP1 is used, the concentration of NU1025

necessary for 50% recovery of the growth inhibition by PARP1 is  $16.5 \pm 1.2 \mu\text{M}$ , and a coefficient of variance (CV value) therefor is 7.3%. It is evident from the above facts that in the method of screening a physiologically active substance using a respiration ability-deficient strain of a yeast, the experimental data obtained hardly show any experimental errors, which makes them remarkably reliable.

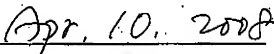
It is evident from the above results that the evaluation system of a physiologically active substance of the present invention using a respiration ability-deficient strain of a yeast exhibits, as compared with the evaluation system of a physiologically active substance using a wild-type strain of a yeast, especially excellent effects that would not in any way be expected from the method using the wild-type strain.

**Statement Under 18 U.S.C. § 1001**

The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

A handwritten signature in cursive script, appearing to read "Masao Tokunaga", written over a horizontal line.

Masao TOKUNAGA

A handwritten date "Apr. 10. 2008" written over a horizontal line.

Date